



The old sheep: a convenient and suitable model for senile osteopenia

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Abstract

Introduction Existing osteoporosis models in sheep exhibit some disadvantages, e.g., challenging surgical procedures, serious ethical concerns, failure of reliable induction of substantial bone loss, or lack of comparability to the human condition. This study aimed to compare bone morphological and mechanical properties of old and young sheep, and to evaluate the suitability of the old sheep as a model for senile osteopenia.

Materials and methods The lumbar vertebral body L3 of female merino sheep with two age ranges, i.e., old animals (6–10 years; $n=41$) and young animals (2–4 years; $n=40$), was analyzed concerning its morphological and mechanical properties by bone densitometry, quantitative histomorphometry, and biomechanical testing of the corticalis and/or central spongious region.

Results In comparison with young sheep, old animals showed only marginally diminished bone mineral density of the vertebral bodies, but significantly decreased structural (bone volume, -15.1% ; ventral cortical thickness, -11.8% ; lateral cortical thickness, -12.2%) and bone formation parameters (osteoid volume, osteoid surface, osteoid thickness, osteoblast surface, all -100.0%), as well as significantly increased bone erosion (eroded surface, osteoclast surface). This resulted in numerically decreased biomechanical properties (compressive strength; -6.4%).

Conclusion Old sheep may represent a suitable model of senile osteopenia with markedly diminished bone structure and formation, and substantially augmented bone erosion. The underlying physiological aging concept reduces challenging surgical procedures and ethical concerns and, due to complex alteration of different facets of bone turnover, may be well representative of the human condition.

Keywords Senile osteopenia · Senile osteoporosis · Large animal model · Old sheep

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Introduction

Osteoporosis is characterized by progressive, systemic bone loss, and micro-architectural changes. The criteria of the World Health Organization for osteoporosis are based on the reference for bone mineral density (BMD) in young adults (age 20–29). While osteoporosis is defined as a BMD ≥ 2.5 SD below this reference, a BMD value more than 1 but less than 2.5 SD below this value is referred to as osteopenia [1, 2]. Additional more recent references recommend to include postmenopausal women and men over the age of 50 years in the diagnosis of osteoporosis, who have a BMD < 2.5 SD below the mean BMD in young adults, but have either experienced a low-trauma hip fracture or a low-trauma vertebral, proximal humerus, pelvis or distal forearm fracture

Table 1 Sheep models for osteoporosis in the lumbar spine

Model	Surgical procedure/ costs	Handling/running costs	Ethical concerns	Extent of bone loss				
				Parameter	3 months	6 months	12 months	24 months
OVX	Simple/low	Simple/low	Medium	BMD	- 1 to - 3% [10, 12]	+ 2 to - 6% [10, 12]	- 2 to - 23% [13, 14, 16]	- 20% [16]
				BV/TV	-	- 11% [14]	- 15 to - 21% [14, 16]	- 7 to - 21% [15, 16]
				Tb.Th	-	- 7% [14]	- 20% [14, 16]	- 14 to - 20% [15, 16]
				Strength	-	+ 2% [14]	- 10 to - 45% [13, 14]	-
Diet	-	Medium/low	Medium	BMD	-	- 12% [11]	-	-
				BV/TV	-	- 35% [19]	-	-
				Tb.Th	-	- 29% [19]	-	-
				Strength	-	- 20% [19]	-	-
Steroids	-	Medium/low	High	BMD	- 10% [12]	- 7 to - 15% [11, 18]	-	-
				BV/TV	-	+ 2% [18]	-	-
				Tb.Th	-	+ 0% [18]	-	-
				Strength	-	- 31% [18]	-	-
OVX+S	Simple/low	Medium/low	High	BMD	-	- 51% [18]	-	-
				BV/TV	-	- 38% [18]	-	-
				Tb.Th	-	- 30 to - 58% [18, 21]	-	-
				Strength	-	- 29 to - 82% [18, 21]	-	-
OVX+D+S	Simple/low	Medium/medium	High	BMD	- 21% [20]	- 33 to - 63% [18, 21]	-	-
				BV/TV	- 22% [20]	-	-	-
				Tb.Th	- 27% [20]	-	-	-
				Strength	- 23% [17]	-	-	-
OVX+leptin	Demanding/high	Demanding/high	High	BV/TV	- 15% [17]	-	-	-
				BMD	- 7% [24]	- 24% [24]	- 48% [15]	
				BV/TV	- 8% [24]	- 21% [24]	- 28% [15]	
				BMD	- 3.7%	-	-	
OVX+HPD	Demanding/high	Simple/low	High	BV/TV	- 15.1%	-	-	-
				Tb.Th	- 5.4%	-	-	-
				Cort.Th	- 11.8% (ventral), - 12.2% (lateral)	-	-	-
				Strength	- 6.4%	-	-	-

OVX ovariectomy, D diet, S steroids, HPD surgical disconnection of the hypothaloma-pituitary axis, BMD bone mineral density, BV/TV bone volume/total volume, Tb.Th trabecular thickness, Cort.Th cortical thickness

in combination with an osteopenia diagnosis by diminished BMD (> 1 SD and < 2.5 SD [1]).

The development of new drugs or implant devices in the context of osteoporosis requires suitable animal models. The United States Food and Drug Administration (FDA) recommends that new therapeutic agents should undergo testing in the ovariectomized (OVX) rat and in a second, non-rodent, large animal model with intracortical bone remodeling [3, 4]. Beside other animals, e.g. dogs, goats or primates, many studies have used sheep for this purpose because of their large size and bone structure, which is comparable to the human situation [5–8]. In addition, sheep are easily available and well affordable concerning purchase and housing. However, BMD and bone mineral content are significantly higher in sheep than in humans. These differences also result in increased mechanical properties of ovine compared to human bone [9, 10]. In addition, ruminants such as sheep differ from humans in their diet and food uptake and, subsequently, the size, digesta flow speed, and microbiota of their digestive compartment [11]; also, in sheep the curvature of the lumbar spine is slightly kyphotic rather than lordotic [12, 13].

As most other mammals, sheep do not undergo spontaneous menopause. Therefore, accelerated bone loss due to estrogen deficiency, the major reason for post-menopausal osteoporosis, is not observed in sheep. As a consequence, ovariectomy (OVX) is frequently used to induce a BMD reduction in these animals (Table 1 [14–20]). MacLeay et al. [16], for example, reported a decreased BMD in the last four lumbar vertebrae bodies 6 months after OVX. In contrast, some authors did not find evidence of an induced bone loss by OVX alone in the lumbar spine [17, 21], possibly due to a compensation of the increased bone resorption by a simultaneously increased bone formation [21]. Thus, ewes treated with isolated OVX may be of only limited suitability as a model for osteoporosis.

In contrast, glucocorticoid therapy in sheep, either alone or in combination with OVX, results in a remarkable bone loss of up to 50% [22] and a substantial decrease in bone mechanical properties (Table 1 [23]). However, ethical concerns due to severe side-effects (e.g., massive infections and hair loss) limit the suitability of this model [9, 22, 23].

Calcium and vitamin D restriction, again either as an isolated model or together with OVX and/or steroid therapy, are also used to induce bone loss [22, 24, 25]. Unfortunately, strict dietary regimes require housing of the sheep in cages or ‘in-house’, limiting the well-being of the animals and increasing the costs. Other approaches address the central regulation of the bone metabolism, e.g. by intracerebroventricular application of leptin [21, 26], surgical disconnection of the hypothalamic-pituitary axis [19, 27, 28] or melatonin deficiency caused by surgical pinealectomy (Table 1 [29]). These models induce

significant and reliable bone loss, but are very complex, only partially comparable to human post-menopausal or senile osteoporosis, and require challenging and ethically problematic surgical procedures.

The aim of the present study was therefore to compare the morphological parameters and mechanical properties of the bone in lumbar vertebral bodies of young and old sheep and to evaluate the suitability of the old sheep as a model for senile osteoporosis/osteopenia.

Materials and methods

Animals, surgical procedure, and test specimen

Female merino sheep of two different age ranges were used (young animals: 2–4 years, $n=40$; old animals: 6–10 years, $n=41$). “Sample size determination for diagnostic accuracy studies involving binormal ROC curve indices” [30, 31] for the bone structure parameters bone volume/total volume (BV/TV) and osteoid thickness (O.Th), as well as the bone erosion parameter eroded surface/bone surface (ES/BS) confirmed that sample sizes between 8 and 33 animals per group were sufficient to detect differences with an alpha error probability of 0.05, a power ($1-\beta$ error probability) of 0.80, and a small to medium effect size.

The sheep were fed standard animal chow. To avoid seasonal effects on bone structure, formation, and erosion, the animals were sacrificed with a balanced distribution in the four seasons spring, summer, autumn, and winter by intravenous injection of overdosed barbiturate (Pentobarbital™, Essex Pharma GmbH, Munich, Germany) and subsequent application of magnesium sulfate ($MgSO_4$). The lumbar spine was removed using an oscillating bone saw and kept frozen until further use. The lumbar vertebral body L3 was used for characterization. Exclusively animals/vertebral bodies serving as negative controls (untouched vertebral body L3) from unpublished studies concerning the therapy of experimental lumbar osteopenia or osteochondral repair were used to obtain the present comparative data.

Permission was obtained from the governmental commission for animal protection, Free State of Thuringia, Germany (registration number 02-029/14). All experiments were conducted in accordance with the National Institutes of Health (NIH) Guidelines for the Care and Use of Laboratory Animals.

Digital osteodensitometric investigations

Osteodensitometry was conducted using a software-guided digital bone density measuring instrument (DEXA QDR 4500 Elite™; Hologic, Waltham, USA). To allow an

artifact-free osteodensitometry, the lumbar spine was sawn into individual vertebrae and the spinous and transverse processes, as well as the covering and base plates were removed (final height in each case 15 mm). Osteodensitometric measurements were therefore limited to the central, spongy area of the vertebral body. A rectangular region of interest with a defined size of $9 \times 11 \text{ mm}^2$ served as a measuring field.

Histological/histomorphometrical investigations

After cutting the lumbar vertebral bodies into two parts in the lower third of the vertebral body, the analyses were carried out using two different types of histological sections: (1) decalcified paraffin sections stained by hematoxylin–eosin [32]; or (2) plastic-embedded sections obtained by fixation in acetone and dehydration in ascending alcohol series without demineralization. Static histomorphometry of the lumbar vertebral body L3 was performed according to published procedures [33–35]. For this purpose, the samples were embedded in Technovit 9100 following the instructions of the supplier (Heraeus Kulzer, Wehrheim, Germany [36]). Sections were then cut to a thickness of approx. $7 \mu\text{m}$. For static histomorphometry, the sections were stained with trichrome stain according to Masson–Goldner [37].

Static quantitative histomorphometry of each individual vertebral body was performed using a standard microscope (Axiovert 200 M, Carl Zeiss Microimaging GmbH, Oberkochen, Germany) with a 200-fold magnification, and a Merz counting reticule [35].

The following nine parameters were calculated according to the recommendations of the International Committee of the American Society for Bone and Mineral Research (ASBMR) [33, 34]: Bone volume/total volume (BV/TV), trabecular thickness (Tb.Th), trabecular number (Tb.N), osteoid volume (OV/BV), osteoid surface (OS/BS), osteoid thickness (O.Th), osteoblast surface (Ob.S/BS), osteoclast surface (Oc.S/BS), and eroded surface (ES/BS). Detailed information on the calculation of the parameters can be found in the supplementary data (including Fig. S1). In addition, the cortical thickness was measured in the ventral and lateral region of each vertebral body. Due to the lack of 3D-data of the samples such as micro-CT images or 3D serial sections, the parameter trabecular perforation could not be reliably determined [38, 39].

Biomechanical testing

Biomechanical compressive strength measurements were conducted using a universal material testing machine (Kögel, Leipzig, Germany) and the corresponding software FRK Quicktest 2004.01 (Kögel).

For biomechanical testing, frozen cancellous bone cylinders (10 mm diameter \times 15 mm height) were obtained from

the central part of the vertebral bodies using a surgical diamond hollow milling cutter (10 mm diameter). After a defined thawing time of exactly 30 min, samples were semiconfined in two semilunar clamps (minimal inner diameter of 10.1 mm and length of 9.8 mm) and then compressed along their longitudinal axis until fracturing. This axis was chosen because it represents the main loading axis in humans and is therefore of major interest for future clinical application.

The applied load was recorded in a stress–strain curve until failure and the resulting force was then divided by the surface area of the specimen to obtain the compressive strength in MPa.

The Young's modulus was calculated from the linear region of the stress–strain curve in which the cancellous bone material follows Hooke's law using the formula $E = \frac{\Delta\sigma}{\Delta\varepsilon}$, where $\Delta\sigma$ is the delta of the stress σ and $\Delta\varepsilon$ the delta of the strain ε .

Statistics

Data were expressed as means \pm standard errors of the mean. Differences between groups were analyzed using the Mann–Whitney U test (level of significance $p \leq 0.05$). All statistical tests were performed using the Sigmaplot software release 13.0 (Systat Software Inc., Chicago, USA). “Sample size determination for diagnostic accuracy studies involving binormal ROC curve indices” [30, 31] was used to determine the required sample sizes to detect differences with an alpha error probability of 0.05 and a power ($1 - \beta$ error probability) of 0.80.

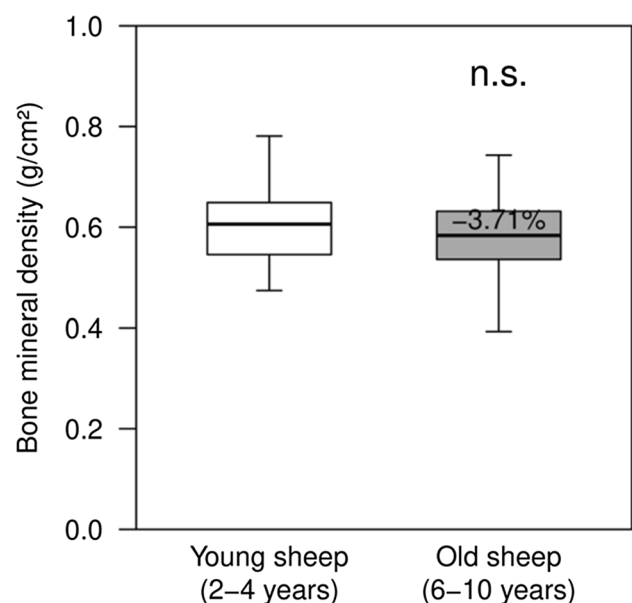
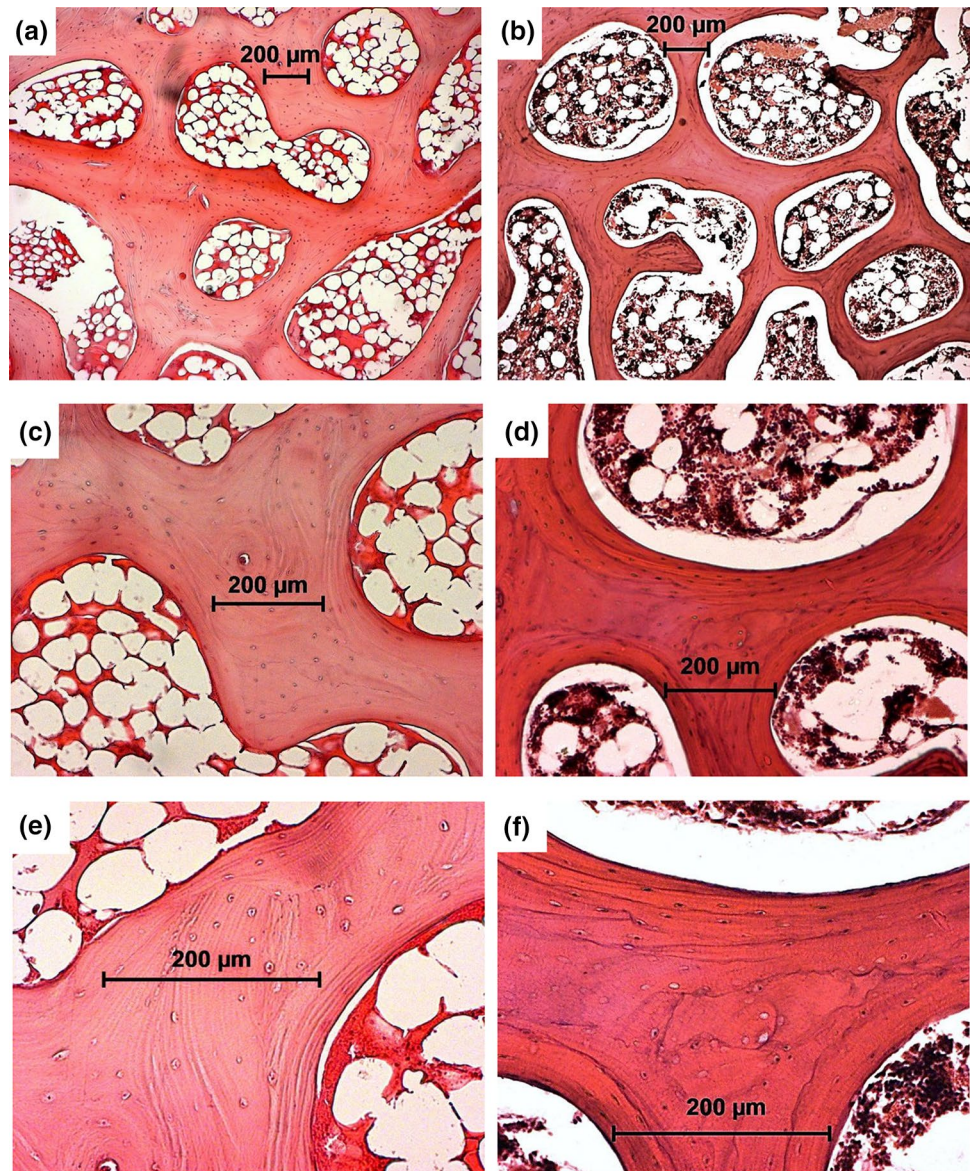


Fig. 1 Bone mineral density of the spongy area in the lumbar vertebral body L3 of young (2–4 years) and old (6–10 years) sheep; n.s. not significantly different vs. young sheep

Fig. 2 Representative hematoxylin eosin staining of the spongy area in the paraffin-embedded lumbar vertebral body L3 of young (2–4 years; **a**, **c**, **e**) and old sheep (6–10 years; **b**, **d**, **f**)



Results

Bone mineral density

The BMD of the central, spongy area of the vertebral bodies in old sheep (6–10 years) was only marginally lower than that in young sheep (2–4 years; decrease of only 3.7%; Fig. 1).

Trabecular bone structural parameters

In contrast, the BV/TV in the lumbar vertebrae of old sheep (Fig. 2b, d, f) was clearly diminished in comparison to that in young sheep (Fig. 2a, c, e). This was confirmed by quantitative histomorphometry, resulting in a

highly significant decrease of the BV/TV ratio in old sheep (– 15.1%; $p \leq 0.001$; Fig. 3a).

The Tb.Th was also numerically decreased in old sheep (– 5.4%; Fig. 3b), while the Tb.N was significantly higher in old sheep (+ 13.4%; $p \leq 0.01$; Fig. 3c).

Cortical bone structural parameters

In parallel to the highly significant decrease of the BV/TV in the spongy area (compare with Fig. 3a), the lumbar vertebrae in old sheep also showed a significant decrease of the thickness of the ventral corticalis (– 11.8%; $p \leq 0.05$; Fig. 4a–c) and the lateral corticalis (– 12.2% $p \leq 0.05$; Fig. 4b–d).

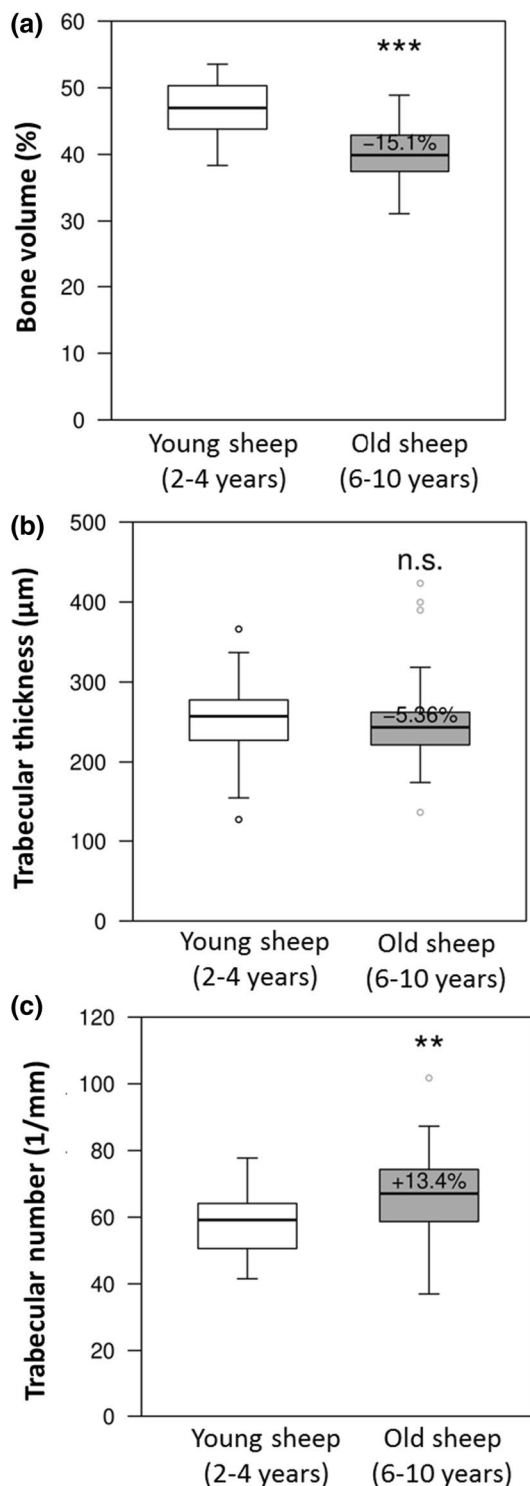


Fig. 3 Structural bone parameters of the spongy area in the lumbar vertebral body L3 of young (2–4 years) and old sheep (6–10 years); **a** bone volume/tissue volume (BV/TV); **b** trabecular thickness (Tb.Th); **c** trabecular number (Tb.N); ** $p \leq 0.01$, *** $p \leq 0.001$ vs. young sheep; n.s. not significantly different vs. young sheep

Trabecular bone formation parameters

Old sheep showed highly significant decreases of the trabecular bone formation parameters OV/BV ($p \leq 0.01$; Fig. 5a), OS/BS ($p \leq 0.001$; Fig. 5b), O.Th ($p \leq 0.001$; Fig. 5c), and Ob.S/BS ($p \leq 0.001$; Fig. 5d; all parameters – 100%).

Trabecular bone erosion parameters

In addition, old sheep displayed substantial and significant increases of the trabecular bone erosion parameters ES/BS ($p \leq 0.01$; Fig. 6a) and Oc.S/BS ($p \leq 0.05$; Fig. 6b).

Biomechanical testing

As a possible consequence of the significantly diminished structural and bone formation parameters (see Figs. 3, 4, 5), as well as the increased bone erosion parameters (Fig. 6), old sheep showed a numerically decreased compressive strength and Young's modulus of the spongy bone cylinders (– 6.4% and – 2.0%, respectively; Fig. 7a, b).

Discussion

Compared to adult young sheep (2–4 years of age), old sheep (6–10 years) showed decreased bone structure and formation (including both trabeculae and corticalis), and increased bone erosion, resulting in a somewhat decreased compressive strength. Physiologically aged sheep may thus qualify as a convenient and suitable model of senile osteopenia with less complex surgical (e.g. ovariectomy), logistic, and ethical challenges than other models. Post-menopausal alterations did not contribute to the changes observed in aged female sheep, since the ewes showed functional ovaries and were all actively cycling, resulting in a model of almost exclusive senile osteopenia free of any interference from the endocrine-metabolic environment of the skeleton (data not shown). Likewise, the presence of ‘laminar’ bone did not influence the results, since neither the young female adult nor the old female sheep analyzed in the present study showed any such laminar bone in histology, but rather exclusively fully developed osteons characteristic of adult bone (compare with Fig. 4 [40, 41]).

In histology, the structural bone parameters in old sheep were moderately, but significantly diminished (decrease of the BV/TV by 15.1% for the spongy area; 11.8–12.2% for the cortical thickness), suggesting a deterioration of the microarchitecture of the lumbar vertebrae in old sheep. On the other hand, the BMD was only marginally decreased in old sheep, possibly due to the limited sensitivity of the technique and the generally higher BMD and bone mineral content in sheep compared to those in humans [9, 10]. The

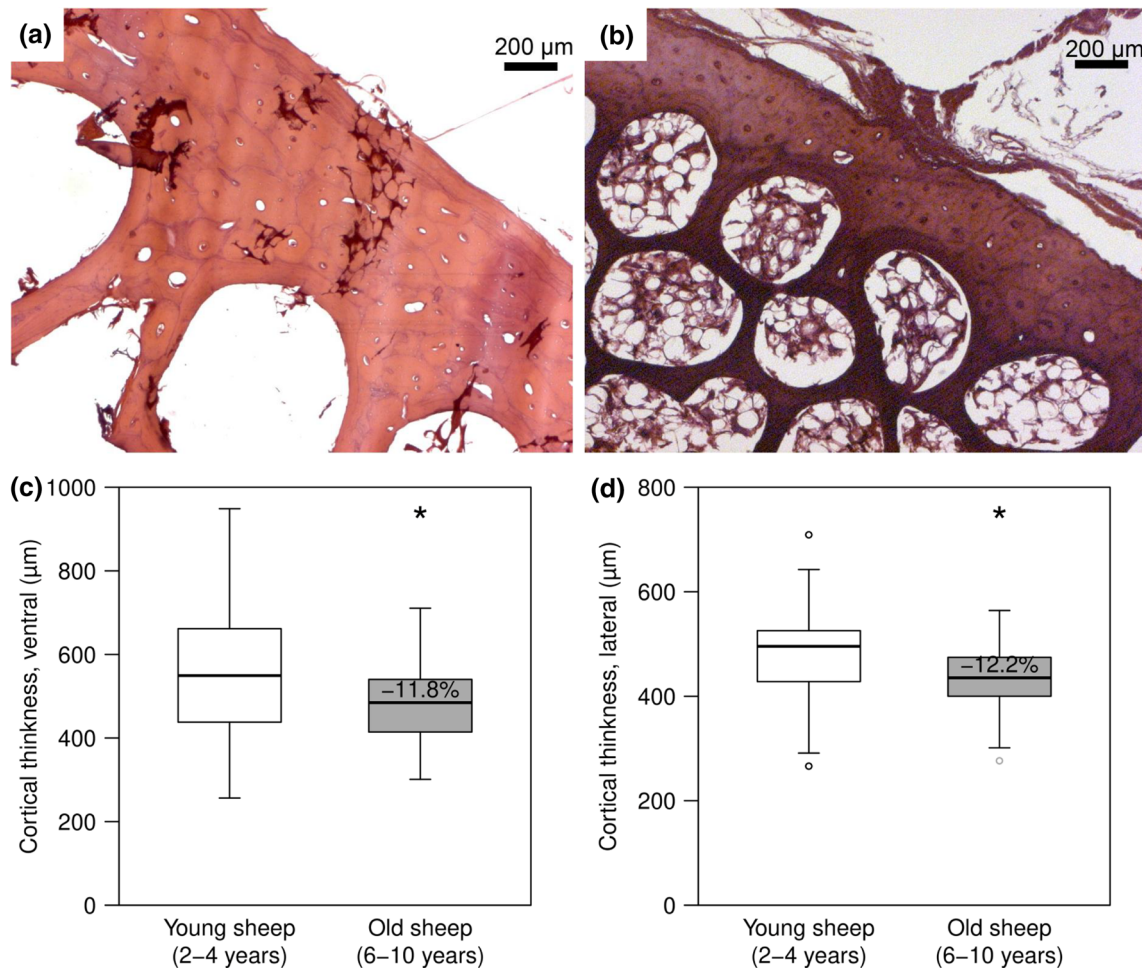


Fig. 4 Representative hematoxylin eosin staining of the corticalis in the paraffin-embedded lumbar vertebral body L3 of young (2–4 years; **a**) and old sheep (6–10 years; **b**); cortical thickness of young and old

sheep in the ventral (**a–c**) and lateral cortical region (**b–d**); * $p \leq 0.05$ vs. young sheep

value of the BV/TV in old sheep was approx. 1.4 standard deviations below that in young sheep, in line with the radiological definition of osteopenia in humans on the basis of the BMD ($-2.5 \text{ SD} < T \text{ score} < -1.4 \text{ SD}$ [1, 2]) according to the diagnostic criteria of the World Health Organization (WHO [42]). Despite a more limited decrease of the structural bone parameters in the present physiological aging model than in other, more substantial models of osteopenia/osteoporosis in sheep (decrease of up to 82%; Table 1), the similarity with human osteopenia may render the current model very attractive for future studies on the pre-clinical therapy of bone pathology. In addition, the model showed a reduced bone structure in both spongiosa and corticalis of the vertebrae, thus fulfilling the recommendations of the FDA for non-rodent, large animal models with intracortical bone remodeling for the testing of new therapeutic agents in bone (patho) physiology [3].

The present sheep osteopenia model was based on substantial, age-dependent changes in both trabecular bone

formation (-100% for all parameters; possibly a strength of the current model) and bone erosion (significant increase for ES/BS and Oc.S/BS), suggesting complex alterations of different facets of bone turnover, as previously described in other more extensive models of sheep osteopenia/osteoporosis (Table 1). The degree of alterations was in the range or even above the changes in other experimental models (Table 1) and the simultaneous alteration of bone formation and erosion underlines the similarity of the current model with the human condition [1, 2].

Compression testing of lumbar vertebrae is recommended for the evaluation of the mechanical properties of cancellous bone [43]. The results of the present study showed a mild to moderate, but merely numerical reduction of compressive strength and Young's modulus in the vertebral bodies of old sheep, indicating that aging in sheep may to some degree reduce bone strength and/or increase bone fragility. This is in line with previous studies demonstrating the sheep's usefulness for osteoporosis research and bone healing due to

Fig. 5 Bone formation parameters of the spongioid area in the lumbar vertebral body L3 of young (2–4 years) and old sheep (6–10 years); **a** osteoid volume/tissue volume (OV/TV); **b** osteoid surface/bone surface (OS/BS); **c** osteoid thickness (O.Th); **d** osteoblast surface/bone surface (Ob.S/BS); ** $p \leq 0.01$, *** $p \leq 0.001$ vs. young sheep

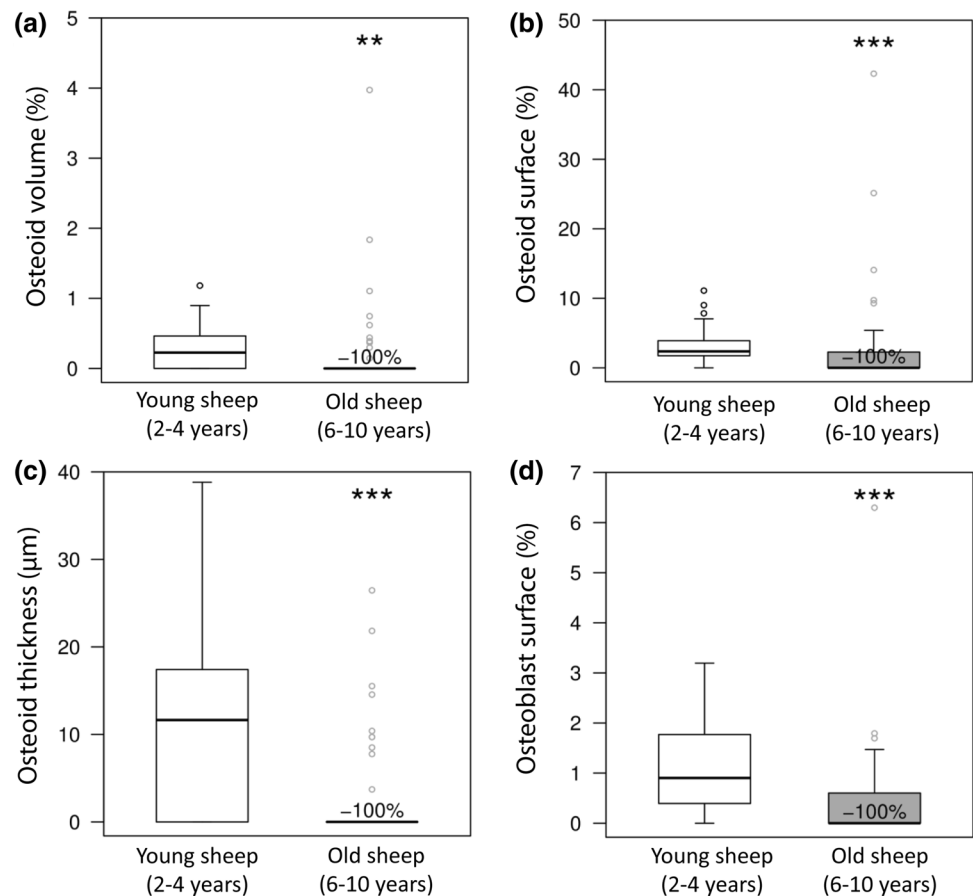
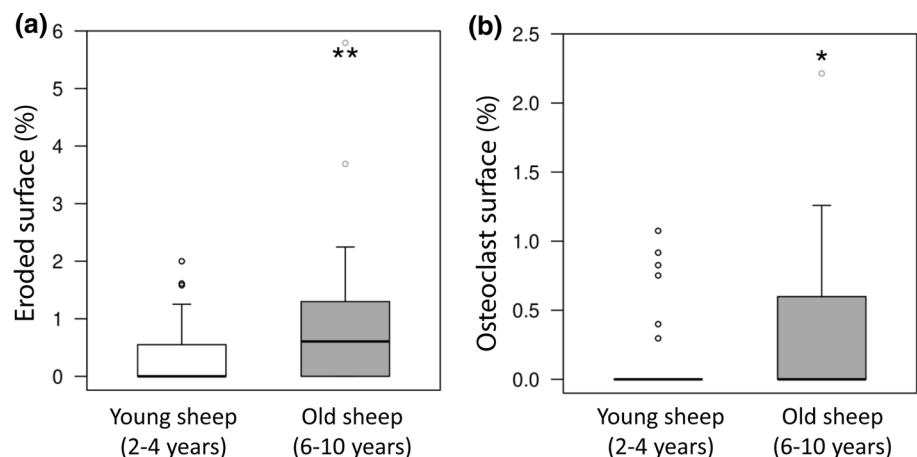


Fig. 6 Bone erosion parameters of the spongioid area in the lumbar vertebral body L3 of young (2–4 years) and old sheep (6–10 years); **a** eroded surface/bone surface (ES/BS); **b** Osteoclast surface/bone surface (Oc.S/BS); * $p \leq 0.05$, ** $p \leq 0.01$ vs. young sheep



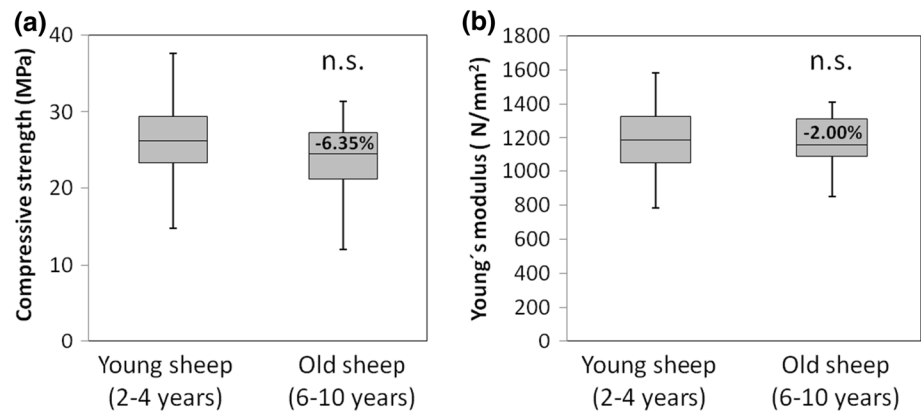
their similarity to humans concerning estrus cycle, hormone profiles, and Harversian bone remodeling [44], as well as the biomechanical properties of the spine ([45] and references therein [46]).

The old sheep has been already successfully used in different published studies as a model for vertebral augmentation with polymethylmethacrylate (PMMA) [47] or with resorbable and osteoconductive calcium phosphate cement (CPC) in minimally invasive lumbar vertebroplasty

[13, 48–51]. The results deriving from the studies with CPC not only validate the old sheep as a valuable model for lumbar osteopenia, but also confirm the potential suitability of such a material to replace the bioinert, non-resorbable and supraphysiologically stiff PMMA currently used in the clinic.

Limitations of the present study include the more limited degree of bone loss during their adult life in sheep (3–10 years) than in humans [10] and the slightly higher

Fig. 7 Compressive strength (a) and Young's modulus (b) of the spongy area in the lumbar vertebral body L3 of young (2–4 years) and old sheep (6–10 years); n.s. not significantly different vs young sheep.



bone density in sheep due to the higher axial compression stresses derived from the quadrupedal locomotion [52]. Another limitation is the lack of an age-matched, ‘healthy’ control population, for example due to the absence of a physiological menopause in sheep, and thus a limited comparability to manifest human osteoporosis. This includes the fact that the ‘*compromised bone strength* predisposing a person to an increased risk of fracture’, which is part of the current NIH ‘definition of osteoporosis’ [53, 54], in the present study does not reach statistical significance when comparing aged and young sheep.

Finally, whereas the simultaneous decrease of both BV/TV and Tb.Th in the present model is in good agreement with the findings in human osteoporosis, the concurrent increase of the Tb.N is not observed in the human disease [11, 12]. On the other hand, this inverse relationship of Tb.Th and Tb.N has been observed previously in aged, ovariectomized sheep [9, 21] and in own studies concerning the augmentation of the bone formation by an injectable CPC through the addition of different BMPs [13, 48–51] and may thus represent a specific feature of bone metabolism in aged sheep.

In comparison to existing sheep osteoporosis models with design, logistic or ethical disadvantages, physiologically aged sheep (6–10 years of age) may provide a suitable model of non-menopausal, senile osteopenia with moderately reduced bone structure, considerably diminished bone formation, and substantially augmented bone erosion.

Despite some limitations concerning the only moderate reduction of the bone structure and the lack of an age-matched ‘healthy’ control population, the model fulfils FDA recommendations for a non-rodent, large animal model with intracortical bone remodeling, and may thus provide a good basis for further mechanistic, diagnostic, and therapeutic studies in bone pathophysiology [3].

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Author contributions SM designed the study and wrote the initial draft of the manuscript. Other authors have contributed to animal care, data collection and interpretation (OB, IH, CB, EK, VH, FG, AS, SB, HS, KDJ, JB). MB and RWK critically reviewed the manuscript. All authors contributed to analysis and interpretation of data, approved the final version of the manuscript, and agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Compliance with ethical standards

Conflict of interest All authors have read the journal’s policy on disclosure of potential conflicts of interest. The authors have no other relevant affiliations or financial or non-financial involvement with any organization or entity with financial or non-financial interest or conflict with the subject matter or materials discussed in the manuscript apart from those disclosed.

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